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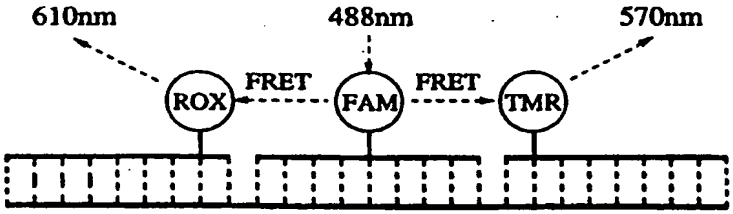
(54) Title: DNA MUTATION MAPPING BY MULTIPLE ENERGY TRANSFER INTERACTIONS



(57) Abstract

A method for determining the presence and location of a mismatch in a target sequence using Fluorescence Resonance Energy Transfer (FRET). The method comprises contacting the target sequence with at least three labelled oligonucleotide probes capable of hybridising to the natural sequence, in juxtaposition. The central probe is labelled with a donor fluorophore and the probe either side of this is labelled with a distinct acceptor fluorophore. Hybridisation to the target sequence results in resonance between the donor fluorophore and the acceptor fluorophores. A mismatch present in the target sequence will disrupt hybridisation to that region, resulting in a significant alteration to the resonance signal.

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DNA MUTATION MAPPING BY MULTIPLE ENERGY TRANSFER INTERACTIONS

Field of Invention

This invention relates to DNA mismatch screening, especially using FRET-detected hybridisation.

Background of the Invention

There is a general increase in the exploration of gene sequences and function. This has generated a need for new approaches to nucleic acid analysis.

One of the simplest methods for detecting gene sequences is to make use of the specific hybridisation reaction between the target sequence and a suitable probe.

Recently, the use of Fluorescence Resonance Energy Transfer (FRET) has been applied to the detection of hybridised probes. Tyagi *et al.*, Nature Biotech. (1996) 14:303-308, describe the use of FRET to distinguish between hybridised and unhybridised probes in a homogeneous assay. The probes each comprise a stem-and-loop structure with the stem formed by the annealing of two complementary arm sequences either side of the probe sequence. A fluorescent moiety is attached to one arm and a non-fluorescent quenching moiety is attached to the other arm. Separation of the stem structure occurs on hybridisation of the probe to the complementary target sequence. This separates the two moieties and allows fluorescence to occur.

Summary of Invention

This invention is based on a realisation of the utility of FRET as a technique for mapping point mutations on a single strand of DNA.

According to the present invention, a method for determining the presence of a mismatch in a target sequence, comprises contacting the target sequence with first, second and third oligonucleotides capable of hybridising to the natural sequence, in juxtaposition, wherein the oligonucleotides are respectively labelled with first, second and third markers having first, second and third absorption wavelengths and first, second and third

emission wavelengths such that there is resonance between either or each of first and second markers and between second and third markers, and observing the presence or absence of each or either resonance.

Description of the Drawings

The accompanying drawings are for the purpose of illustration only. In the drawings:

Fig. 1 is a schematic representation of the interaction of labelled oligonucleotides and target sequence in a screening method embodying the invention;

Fig. 2 is a schematic representation of a more specific embodiment of the invention and represents another arrangement of the labels; and

Fig. 3 illustrates the different emission intensities obtained when point mutations are present in the target sequence, using the arrangement of fluorophores shown in Fig. 2.

Description of Invention

The ability to monitor the interactions between nucleic acids is achieved through the detection of sensitised acceptor emission, due to FRET between a donor fluorophore and two different acceptor fluorophores on associating strands. The absence of any of the components induced by a single base mismatch should cause a detectable loss in sensitised acceptor emission.

The system illustrated in Fig. 1 comprises a target strand and three adjacent complementary oligonucleotides, A, B and C. These are typically each 8-13 base pairs in length. A and B are modified at the 5′ end with a fluorescein moiety (F). B and C are modified at the 3′ end with two distinct types of acceptor fluorophore (X and Y) having different emission maxima and absorption profiles which overlap with the emission profile of fluorescein.

When no mismatches are present, all three oligonucleotides will be bound to the target strand. Thus, when the system is excited at the absorption wavelength of fluorescein (488 nm), FRET will occur and the fluorescence

spectrum will show a decrease in the fluorescein emission relative to each singly-bound probe and appearance of two maxima due to secondary emissions from X and Y. In cases where a mismatch exists somewhere within the target sequence, the region in which the error occurs can be distinguished simply by analysing the fluorescence spectrum. If the mismatch lies within the binding domain of oligonucleotide A, no duplex will form in this region and the secondary emission from X will be lost. Similarly, for mismatches within the B region, the signals from X and Y will disappear, and for errors in region C, the Y emission will be absent.

Fig. 2 illustrates a different arrangement of the fluorophores. Again, three oligonucleotides are used. The central oligonucleotide is labelled with a donor fluorophore, 5'-carboxyfluorescein (fluorescein) only. The two adjacent oligonucleotides are each labelled with an individual acceptor fluorophore, 5'-carboxytetramethyl-rhodamine (TMR) and 5'-carboxyrhodamine-X (ROX). These fluorophores have absorption spectra which overlap with fluorescein's emission spectrum and have well separated emission maxima.

On binding of all three oligonucleotides, the fluorophores are positioned such that excitation of fluorescein results in energy transfer from fluorescein to both TMR and ROX. This transfer can again be monitored by observing the emission of the two acceptor fluorophores. Since the acceptors emit at two distinct wavelengths, introduction of a single base mismatch should be detectable by a loss in either or both of these signals.

The positioning of the fluorophores on each oligonucleotide required to optimise energy transfer can be easily determined by the skilled person by preliminary studies. The fluorophores will typically be separated, e.g. by a distance of 7 bases.

FRET assays can be carried out using a fluorimager, irradiating the assays with a laser at a suitable

wavelength, e.g. 488 nm for fluorescein, and scanning the emissions using suitable filters, e.g. 530 nm for fluorescein, 570 nm for TMR and 610 nm for ROX.

A characteristic of the common, donor fluorophore is that it should emit at a wavelength that is capable of exciting each of the acceptor fluorophores. The emission of each acceptor fluorophore must be resolvable.

An example of a common fluorophore that can be used as a donor is carboxyfluorescein. Additional examples of suitable acceptors include N,N,N',N'-tetramethyl-6-carboxyrhodamine and 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein.

The probes may be of DNA. Alternatively, and on the same principle, fluorescently-labelled DNA mimics, e.g. PNA, phosphorothioate DNA, could be used as the probes.

By the use of additional fluorophores with distinct emission profiles, it may be possible to extend this system for the screening of longer target sequences using multiple short complementary strands. This arrangement may permit screening of a section of the DNA target gene in a single homogeneous assay by excitation with, e.g. a fixed wavelength laser, or a fluorescence spectrometer, and instantly generating an emission spectrum which characterises where, if anywhere, there is a point mutation or damage. It is envisaged that sequences of diagnostic oligonucleotides may be chosen to generate diagnostic kits for screening regions of mutational "hotspots".

The following Example illustrates the invention. In particular, it shows the ability to detect and locate point mutations in a target oligonucleotide.

Example

Four target oligonucleotides (SEQ ID Nos 1-4) were designed, each differing only in one nucleotide.

SEQ ID No 1 5'CGTTCTAAGGATTACGTCGAACCTTTG3'

SEQ ID No 2 5'CGTTCAAAGGATTACGTCGAACCTTTG3'

SEQ ID No 3 5'CGTTCTAAGGATAACGTCGAACCTTTG3'

SEQ ID No 4 5'CGTTCTAAGGATTACGTCGAACCATTG3'

Using oligo SEQ ID No 1 as the control, three oligonucleotide probes (SEQ ID No. 5-7) were designed, each capable of hybridising to a distinct region on the control.

SEQ ID No 5 3'GCAAGAT*TC5'

SEQ ID No 6 3'CTAA*TGCAG5'

SEQ ID No 7 3'CT*TGGAAAC5'

The probes were labelled with a fluorophore at the positions marked (*) as follows: probe SEQ ID No 5 with ROX; probe SEQ ID No 6 with fluorescein; and probe SEQ ID No 7 with TMR.

The emission intensity for both ROX and TMR-labelled probes increased significantly (relative to each singly-bound probe) on hybridisation to the control. However, as shown in Fig. 3, on reaction with each of the target oligonucleotides SEQ ID Nos 2-4, the emission intensity varied significantly, depending on the position of the single nucleotide difference from the control.

This assay provides a simple method for the analysis of hybridisation events in solution and enables detection of mutations within defined regions of a target strand from a single fluorescence measurement. The assay may be modified to utilise immobilised target or probes.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Cambridge University Technical Services Ltd.
(B) STREET: The Old Schools, Trinity Lane
(C) CITY: Cambridge
(D) STATE: N/A
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): CB2 1TS

(ii) TITLE OF INVENTION: DNA MUTATION MAPPING BY MULTIPLE ENERGY TRANSFER INTERACTIONS

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: WO Not yet known

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CGTTCTAAGG ATTACGTCGA ACCTTTG 27

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CGTTCAAAGG ATTACGTCGA ACCTTTG 27

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGTTCTAAGG ATAACGTCGA ACCATTG 27

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CGTTCTAAGG ATTACGTCGA ACCATTG 27

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCAAGATTC 9

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CTAATGCAG 9

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CTTGGAAAC 9

CLAIMS

1. A method for determining the presence and location of a mismatch in a target sequence, which comprises contacting the target sequence with first, second and third oligonucleotides capable of hybridising to the natural sequence, in juxtaposition, wherein the oligonucleotides are respectively labelled with first, second and third markers capable of absorption at first, second and third wavelengths and emission at first, second and third wavelengths, such that there is resonance between either or each of first and second markers and between second and third markers, and observing the presence or absence of resonance.

2. A method according to claim 1, wherein the second oligonucleotide comprises two second markers resonating with the first and third markers respectively.

3. A method according to claim 1 or claim 2, wherein one or more markers is a fluorescent moiety.

4. A method according to any preceding claim, wherein the one or more markers are selected from 5'-carboxyrhodamine-X, 5'-carboxytetramethylrhodamine, N,N,N',N'-tetramethyl-6-carboxyrhodamine, 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein and 5'-carboxyfluorescein.

5. A method according to any preceding claim, wherein the oligonucleotides each comprise 8-13 bases.

6. A method according to any preceding claim, wherein adjacent markers are separated by at least 7 bases.

7. A method according to any preceding claim, wherein the target or the first, second and third oligonucleotides are immobilised.

8. A kit comprising, in separate compartments, first, second and third markers defined in any of claims 1 to 5.

FIG. 1

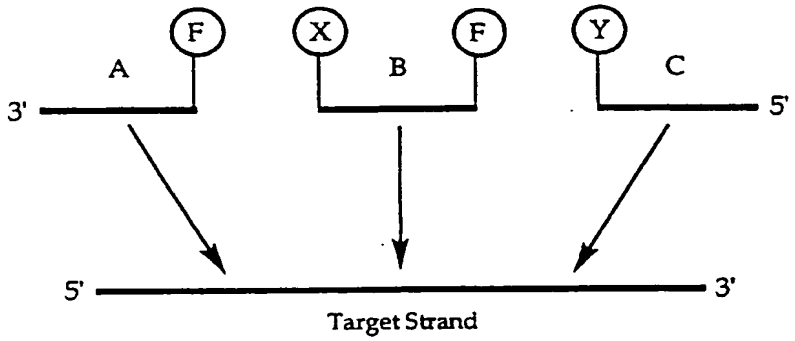
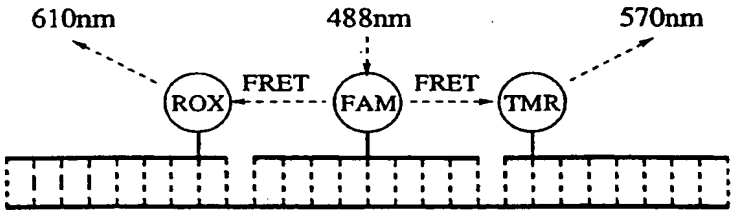


FIG. 2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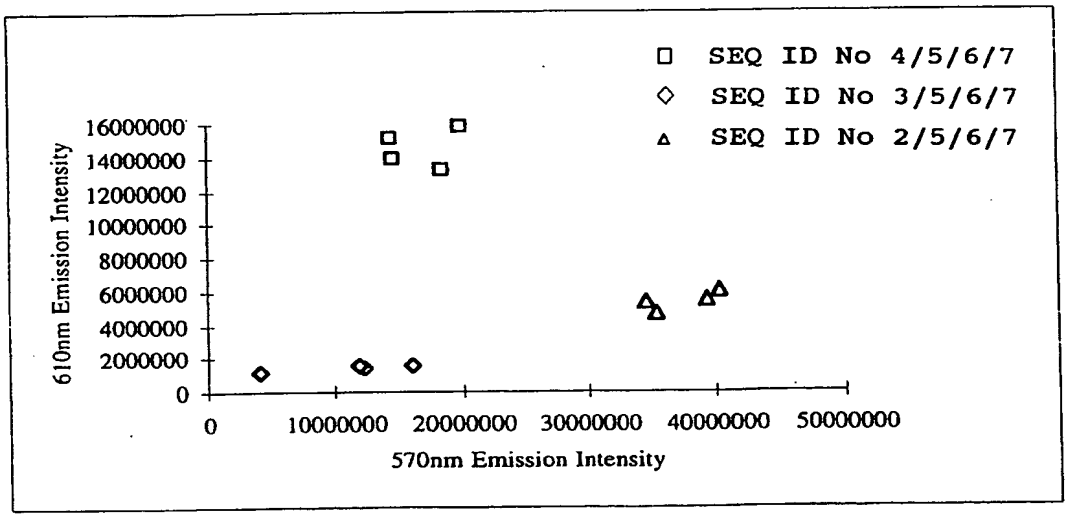
488nm
570nm
FRET
FRET
ROX
FAM
TMR

FIG. 3


SEQ ID No 4/5/6/7
SEQ ID No 3/5/6/7
SEQ ID No 2/5/6/7
610nm Emission Intensity
16000000
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
0
10000000
20000000
30000000
40000000
50000000
570nm Emission Intensity

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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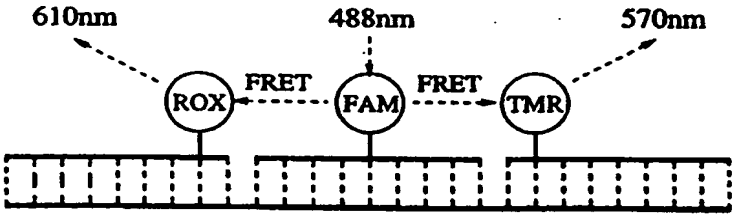

(57) Abstract

A method for determining the presence and location of a mismatch in a target sequence using Fluorescence Resonance Energy Transfer (FRET). The method comprises contacting the target sequence with at least three labelled oligonucleotide probes capable of hybridising to the natural sequence, in juxtaposition. The central probe is labelled with a donor fluorophore and the probe either side of this is labelled with a distinct acceptor fluorophore. Hybridisation to the target sequence results in resonance between the donor fluorophore and the acceptor fluorophores. A mismatch present in the target sequence will disrupt hybridisation to that region, resulting in a significant alteration to the resonance signal.

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INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/GB 98/01138

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 25518 A (SOCIETY FOR TECHNO INNOVATION ;MASUKO MASAYUKI (JP); EBATA KATSUYO) 22 August 1996 see figure 3 & EP 0 810 291 A see column 3, line 41 - line 47; figure 3	1-8
X	EP 0 229 943 A (MOLECULAR BIOSYSTEMS INC) 29 July 1987 see page 13, last paragraph; figure 5	1-8
A	WO 93 09128 A (NANOTRONICS INC) 13 May 1993	

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of theinternational search	Date of mailing of the international search report
16 October 1998	26/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Molina Galan, E

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INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/GB 98/01138

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TYAGI S ET AL: "MOLECULAR BEACONS: PROBES THAT FLUORESCE UPON HYBRIDIZATION" BIO/TECHNOLOGY, vol. 14, 1 March 1996, pages 303-308, XP000196024 cited in the application	
A	EP 0 601 889 A (MAINE MEDICAL CENTER RES) 15 June 1994	

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ,onal Application No
PCT/GB 98/01138

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		EP	0810291 A	03-12-1997
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